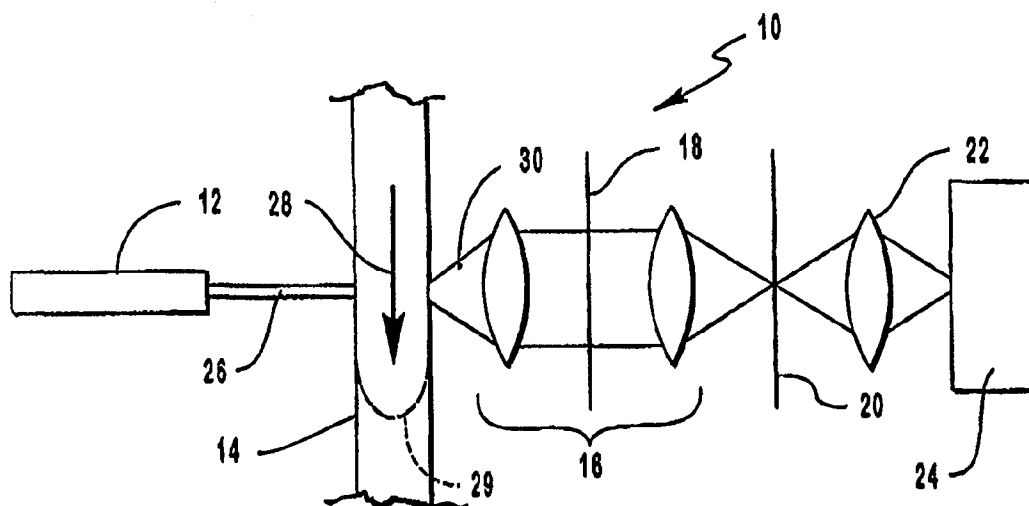


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) <b>International Patent Classification</b> <sup>7</sup> :  <b>G01N 15/02</b></p>	<p><b>A1</b></p>	<p>(11) <b>International Publication Number:</b> <b>WO 00/42412</b></p> <p>(43) <b>International Publication Date:</b> 20 July 2000 (20.07.00)</p>
<p>(21) <b>International Application Number:</b> PCT/US00/00573</p> <p>(22) <b>International Filing Date:</b> 11 January 2000 (11.01.00)</p> <p>(30) <b>Priority Data:</b>  09/228,247 11 January 1999 (11.01.99) US</p> <p>(71) <b>Applicant:</b> SOFTRAY, INC. [US/US]; 519 South Fifth Street, Laramie, WY 82070 (US).</p> <p>(72) <b>Inventor:</b> JOHNSON, Paul; 519 South Fifth street, Laramie, WY 82070 (US).</p> <p>(74) <b>Agents:</b> HOLMES, John et al.; Roylance, Abrams, Berdo &amp; Goodman, LLP, Suite 600, 1300 19th Street, N.W., Washington, DC 20036 (US).</p>		<p>(81) <b>Designated States:</b> JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b>  <i>With international search report.</i></p>

**(54) Title:** IMPROVED FLOW CYTOMETRY APPARATUS AND METHOD



**(57) Abstract**

A flow cytometer (14) for detecting target particles such as microorganisms including biological cells and viruses as well as molecular species. The flow cytometer includes a detection system (10) involving a CCD (24) having a time delay integration capability to thereby increase the signal from the target particle (33) and decrease the noise detected by the CCD. Calibration particles (48) can be included in the sample stream of the flow cytometer (28) for coordinating the readout of the CCD with the rate of flow of the sample stream to improve the detection capability of the CCD. Statistical analysis techniques can also be used to determine the rate of flow of target particles in the sample stream to thereby coordinate the readout rate of the CCD with the rate of flow of the target particle.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## IMPROVED FLOW CYTOMETRY APPARATUS AND METHOD

BackgroundField of the Invention

This invention relates to flow cytometry and, more particularly, to an improved flow cytometry apparatus and method involving a novel time delayed integration of a charge coupled device in the detection apparatus for enhancing the signal-to-noise ratio.

The Prior Art

Cytometry is the measurement of the physical and/or chemical characteristics of biological cells. Since the technology of my novel discovery as disclosed and claimed herein can be adapted to other systems such as viruses and molecules as well as biological cells, I have adopted the term "cytometry" to refer to the detection of all of these species which, for the sake of convenience are referred to herein as "target particle(s)." Flow cytometry, as the name implies, involves the delivery of a flowing stream containing a sample having target particles therein to the detection region of a flow cytometer. The target particles in the detection region are irradiated using a laser to create an illumination phenomena by the target particles. The appropriate optics and detection electronics measure the light absorption; scattering; fluorescence; and/or spectral properties of the target

1 particles in the sample, or alternatively, a fluorescent label  
2 affixed to the target particles. The laser is typically a gas  
3 laser (such as an Argon or a Helium Neon Laser) or even a diode  
4 laser. In the case of fluorescence, each target particle produces  
5 a burst of fluorescence photons as it passes through the  
6 illumination region. Differentiation of the fluorescence from the  
7 illumination or excitation light can be accomplished with a filter  
8 or a combination of filters. Detection of the fluorescence is  
9 accomplished using a photomultiplier tube or a photodiode. Another  
10 technique relies on light scattering of photons in the illumination  
11 beam by the target particle. The target particle is identified by  
12 its light scattering as a function of the angle of scattering which  
13 is a function of its size and shape as well as the wavelength of  
14 the scattered photons.

15 The successful detection and identification of a single target  
16 particle depends upon several factors. First, the laser power must  
17 be sufficient to generate a large enough number of fluorescence  
18 (or, alternatively, scattering) photons during the brief passage of  
19 the target particle through the irradiation region. Specifically,  
20 it is essential that a sufficient number of photons are generated  
21 so that the fluorescent burst from the target particle can be  
22 reliably differentiated from random fluctuations in the number of  
23 background photons. Second, it is important to minimize these  
24 unwanted background photons. These unwanted background photons  
25 arise from scattering or from fluorescence emitted by the carrier  
26 liquid of the sample or impurities in the liquid, as well as from

1 the apparatus itself, such as the walls of the capillary through  
2 which the flow stream passes.

3 Flow cytometry inherently creates competition between a  
4 desirable high flow rate for a reasonable sample throughput in  
5 order to provide rapid detection of target particles and high  
6 detectivity. High detectivity is predicated upon having a  
7 fluorescing (or scattering) target particle in the detection beam  
8 long enough to provide a high signal-to-noise detection along with  
9 an optical design that will minimize background photons from  
10 unwanted scattering and unwanted fluorescence, both of which can  
11 dominate system noise. Optimally, a flow cytometer will have a  
12 small excitation beam, a high flow rate, and detection optics and  
13 associated electronics that allow for the collection of sufficient  
14 photons from the target particle to provide high signal-to-noise  
15 detection.

16 Various flow cytometry devices are described in available  
17 references. See, for example Flow Cytometry: First Principles,  
18 A.L. Givan (1992). Other references include Sage et al. (U.S.  
19 Patent No. 4,660,971); Wu et al. (U.S. Patent No. 4,661,913);  
20 Yamamoto et al. (U.S. Patent No. 4,786,165); Ohki et al. (U.S.  
21 Patent No. 5,007,732); and Hirako (U.S. Patent No. 5,135,302).  
22 Each of these references also contain references to other relevant  
23 publications.

24 As discussed previously, the competing requirements imposed on  
25 flow cytometry create an inherent conflict between high sample  
26 throughput and high detectivity that has proven most difficult to

1 overcome resulting in limitations in applications for flow  
2 cytometry. In view of the foregoing, it would be an advancement in  
3 the art to provide improvements in flow cytometry. It would be  
4 another advancement in the art to provide a charge coupled device  
5 (CCD) as the detector with the image from the target particle being  
6 shifted across the CCD at the same rate that the CCD charge is  
7 being shifted across the CCD. It would be an even further  
8 advancement in the art to provide a flow cytometer with a time  
9 delayed integration (TDI) system to significantly alter the signal-  
10 to-noise ratio in favor of detection. Such a novel apparatus and  
11 method is disclosed and claimed herein.

#### 12 13 Brief Summary and Objects of the Invention

14 This invention involves the detection of target particles such  
15 as microorganisms, molecules, and the like, using a CCD to provide  
16 TDI of the photons from the target particle in the sample stream of  
17 the flow cytometer. TDI provides a surprisingly high signal-to-  
18 noise ratio of the detection photons by lowering the contribution  
19 of photons from unwanted background photons with respect to the  
20 photons emitted by the target particle and by lowering the  
21 contribution of CCD readout noise. This high signal-to-noise ratio  
22 is accomplished by effectively narrowing the field of view thereby  
23 reducing the contribution by the background illumination. CCD  
24 spectrophotometry can also be used to detect spectra from the  
25 target particle to measure the properties of the target particle as  
26 well as being able to differentiate between the scattering and

1 fluorescence photons from the target particle. Further, TDI of  
2 flow cytometry using a CCD accommodates simultaneous detection and  
3 analysis of multiple target particles.

4 It is, therefore, a primary object of this invention to  
5 provide improvements in detection of target particles using flow  
6 cytometry.

7 Another object of this invention is to provide improvements in  
8 the method of identifying target particles using flow cytometry.

9 Another object of this invention is to provide a flow  
10 cytometry analysis of target particles with TDI.

11 Another object of this invention is to use TDI to enhance the  
12 signal to noise ratio of a flow cytometry apparatus.

13 Another object of this invention is to use TDI in combination  
14 with a flow cytometry apparatus to provide for the simultaneous  
15 detection and analysis of multiple target particles in the sample  
16 stream.

17 Another object of this invention is to coordinate the rate of  
18 movement of the image across the CCD during TDI of the CCD with the  
19 rate of flow of the target particles in the sample flow.

20 Another object of this invention is to provide for the facile  
21 correlation between the scattering and fluorescence signals from  
22 the target particles.

23 These and other objects and features of the present invention  
24 will become more readily apparent from the following description in  
25 which preferred and other embodiments of the invention have been

1 set forth in conjunction with the accompanying drawing and appended  
2 claims.

3  
4 Brief Description of the Drawing

5 Figure 1 is a schematic of a first embodiment of an imaging  
6 TDI/CCD flow cytometry apparatus of this invention;

7 Figure 2 is an enlarged, schematic illustration of two target  
8 particles in the core flow of the TDI/CCD flow cytometer of Figure  
9 1 as they pass through the excitation zone of a laser beam;

10 Figure 3 is an enlarged schematic illustration of the face of  
11 a CCD illustrating the charge shift on the CCD at the same velocity  
12 as the flow velocity of the target particles;

13 Figure 4 is a schematic of a second embodiment of a TDI/CCD  
14 flow cytometry apparatus of this invention for detecting two or  
15 more target particles having different emission spectra;

16 Figure 5 is an enlarged, schematic illustration of two target  
17 particles having different emission spectra passing through the  
18 flow zone of the TDI/CCD flow cytometer of Figure 4; and

19 Figure 6 is an enlarged schematic illustration of the face of  
20 a CCD illustrating the charge shift on the CCD from the two target  
21 particles of Figure 5.

22  
23 Detailed Description of the Preferred Embodiment

24 The invention is best understood from the following  
25 description with reference to the drawing wherein like parts are



1 designated by like numerals throughout and taken in conjunction  
2 with the appended claims.

#### 3 4 General Discussion

5 A CCD is a solid state device designed to act as an  
6 "artificial retina." A dense array of light receptors or pixels  
7 are formed over the surface of the CCD. Light striking a pixel  
8 creates an electronic charge proportional the intensity or number  
9 of photons in the light that strike the pixel during exposure.  
10 Conventionally, a CCD image of an object is created by opening a  
11 shutter and exposing the CCD to the object. The shutter is then  
12 closed and the CCD is read by transferring each charge, row by row,  
13 into a serial transport register. After each row transfer, the  
14 individual pixels are transferred, one at a time, through an on-  
15 chip output amplifier and digitized. The readout and digitization  
16 are generally performed as rapidly as possible, restrained only by  
17 the existing electronic noise conditions.

18 The coupling of TDI techniques to a CCD generally requires  
19 each row to be shifted more slowly in order to synchronize the  
20 pixel shift rate with the rate at which the image (of target  
21 particles in the flow stream of the flow cytometer) moves across  
22 the CCD. The flow cytometry CCD is not shuttered as described  
23 above but is read out on a continuous basis. Image smearing is  
24 avoided by moving the image across the CCD at the same rate that  
25 the CCD charge is being shifted across the CCD. Accordingly, the  
26 charge imposed on each pixel that is read out will have dwelt for

1 an equal period of time on each pixel in one column of the CCD.  
2 This means that all of the image pixels read from a single column  
3 of the CCD will have been detected with nearly equal quantum  
4 efficiency, the mean quantum efficiency of the CCD column.

5 When used for improving the signal-to-noise ratio for imaging,  
6 TDI will permit one to obtain a signal-to-noise many times higher  
7 than when using a single detector such as a conventional  
8 photomultiplier tube. This means that stray photons from the  
9 background are significantly minimized when compared to the overall  
10 signal from the target particle. For example, one could use a  
11 conventional photomultiplier tube to detect emission from a 1  $\mu\text{m}$   
12 bacteria in a column of liquid with an illuminated length of 200  $\mu\text{m}$   
13 and a 50  $\mu\text{m}$  square cross section. Alternatively, one could use a  
14 CCD with 9  $\mu\text{m}$  square pixels. If a bacterium is contained in a  
15 single pixel the background contribution would be reduced by over  
16 a factor of more than 100. Clearly, of course, these dimensions  
17 and numerical specifications are representative and are provided  
18 herein for illustrative purposes only.

19 The importance of this invention is more clearly understood  
20 when one considers the fact that the primary source of noise in a  
21 flow cytometer is the high level of laser emission scattered by the  
22 carrier fluid, particles in the sample flow, and the components of  
23 the flow cytometer itself. A notch filter at the laser line  
24 frequency can reduce the intensity of this background scattering by  
25 a factor of about  $10^{-5}$ , but even then the fluorescence from unbound  
26 dye molecules (in an immunofluorescence flow cytometer, for

1 example) can readily overwhelm bacterial fluorescence in a single  
2 detector flow cytometer. In conventional flow cytometry this  
3 problem is addressed by illuminating and collecting light from only  
4 a very small region of the flow. On the other hand, TDI achieves  
5 the same result without the need to restrict the illuminated  
6 volume. A much larger illuminated volume can be used, resulting in  
7 a longer residence time for each target particle in the laser beam  
8 and a corresponding increased number of collected detection  
9 photons.

10 TDI also allows one to image a small volume of sample flow  
11 surrounding an individual target particle without knowing exactly  
12 where the target particle resides inside the volume. When the  
13 emission of a laser-illuminated flow cell is imaged onto a CCD with  
14 the direction and rate of flow aligned with the column direction of  
15 the CCD chip, the CCD may be read out at a rate such that the  
16 fluorescence emission from a single target particle always  
17 accumulates in a single moving charge packet on the CCD chip.  
18 Ideally, emitted light from a single target particle will reside in  
19 a single pixel.

20 In the best case, emitted light from the target particle will  
21 reside in a single pixel region on the CCD. Accordingly, the  
22 benefit of TDI/CCD imaging over a single pixel detector is that the  
23 contribution of background photons from scattered light and  
24 fluorescence from unbound dye molecules can be decreased by several  
25 orders of magnitude, depending on the diameter of the sample

1 stream, the CCD pixel size, and the magnification capability of the  
2 system.

3       Additionally, TDI has benefits over traditional CCD imaging,  
4 where multiple short-exposure frames might be used to track a  
5 target particle moving through an illuminated flow cell. The CCD  
6 and its associated electronics contribute a quantity of noise,  
7 called the readout noise, each time a pixel is read from the  
8 device. In TDI mode a single pixel, optimally, will follow a  
9 single target particle. When the pixel is digitized it will  
10 contain one contribution of readout noise. For illustration we  
11 assume a readout noise of 100 electrons rms for a single pixel. If  
12 non-TDI imaging is used and 100 short-exposure images are used to  
13 follow a target particle through the flow and said images are  
14 combined by statistical means, the combined contribution from  
15 readout noise is the square root of  $100 \times 10$  electrons rms, or 100  
16 electrons rms. Also, TDI is less computationally intensive than  
17 using multiple short-exposure images.

18       As is apparent from the foregoing, one of the primary  
19 requirements for using TDI with CCD flow cytometry is the need to  
20 accurately synchronize the TDI with the flow rate in the flow  
21 cytometer. A differential between the flow rate and the TDI will  
22 result in streaks appearing in the CCD "images." That is, the  
23 emitted light from a target particle will extend over many pixels  
24 along the column direction of the CCD. The flow rate can be  
25 coordinated with the TDI rate using calibration particles in the  
26 sample flow. Calibration particles can also be introduced into the

1 flow during the actual experiment as long as the calibration  
2 particles have a different emission spectrum than the target  
3 particle being detected. Alternatively, sample flow through a  
4 capillary will exhibit a parabolic-shaped velocity profile as a  
5 result of drag imposed on the liquid stream by the surrounding  
6 walls of the capillary which means that one can utilize  
7 conventional statistical techniques to coordinate the TDI of the  
8 CCD with the flow rate in order to minimize streaks in the CCD  
9 images.

10 The simultaneous detection of two or more different species  
11 requires that each species has a unique fluorescing wavelength  
12 and/or scattering phase function. Different emission wavelengths  
13 can occur due to differences in the intrinsic fluorescence (e.g.  
14 autofluorescence or bioluminescence) of the target particles or by  
15 tagging them with chemical labels that have differing emission  
16 wavelengths. Tagging then allows the use of a TDI/CCD  
17 spectrophotometer as a detector that can discriminate between  
18 individual target particles and in real time. This means that the  
19 individual spectra will be associated with rows of charge packets  
20 on the CCD rather than having individual spots (associated with  
21 single charge packets on the CCD) shifted down the CCD in the TDI  
22 imaging mode.

23 Further, since a CCD can be binned, i.e., a rectangular  
24 grouping or "bin" of pixels can be read out as a single superpixel  
25 with a single contribution of readout noise thereby meaning that  
26 the spectral resolution of the CCD can be changed to meet the

1 problem encountered, while minimizing readout noise. Binning also  
2 allows for faster CCD readout, with the readout speed being  
3 approximately proportional to the binning factor. For example, if  
4 the CCD is binned by a factor of ten along the row dimension the  
5 CCD can be read out approximately ten times faster. This means  
6 that for a single-species detection, a 768 x 512 CCD can be read  
7 out as a 768 x 1 device thereby providing for an increase in  
8 readout speed of approximately 500.

#### 9 10 Flow Cytometry Flow Rate Considerations

11 Conventionally, the core diameter or cross sectional area of  
12 the irradiation zone of the flow cytometer determines the volume of  
13 the background solution illuminated by the laser and, therefore,  
14 viewed by the detection system. The rate of solution flow through  
15 this irradiation zone determines the length of time the target  
16 particle is being irradiated by the laser beam. This is referred  
17 to as the transit time. Transit time is important because the  
18 residual, unbound fluorescent tag in the observed volume of  
19 solution contributes to the number of background photons collected  
20 by the detection system. Accordingly, a large number of background  
21 photons can reduce the signal-to-noise ratio to such a degree as to  
22 render detection of the target particle impossible. This means  
23 that the core diameter and the transit time jointly determine the  
24 signal-to-noise ratio for the detection of the target particle.

25 The critical parameter that affects the transit time is the  
26 flow rate of the solution through the irradiation zone. This

parameter is typically designated as the core volumetric flow rate,  $q$ , in milliliters per second, ml/s, and is related to the cross sectional area of the irradiation zone,  $A$ , in square centimeters,  $\text{cm}^2$ , along with the flow velocity,  $v$ , in centimeters per second, cm/s. Accordingly the cross sectional area,  $A$ , can be expressed as  $A = q/v$ . If the irradiation zone is circular then its diameter,  $d$ , in micrometers, can be calculated using the equation  $d = 1.13 \times 10^{-4} (q/v)^{1/2}$ .

As discussed earlier, the time required to read one line of the CCD chip determines the flow rate because the TDI measurement requires the sample flow to be synchronized as closely as possible with the charge transfer on the CCD chip. One low-cost CCD camera that has TDI and binning implemented in software uses a CCD chip with  $9 \mu\text{m}$  square pixels and can be read out at a rate corresponding to a maximum flow rate of 25 cm/s (assuming an image magnification of one). Consideration of the simultaneous needs for spectral dispersion (i.e., approximately 50 spectral elements) and lateral binning on the CCD chip suggests an optimal core diameter of  $50 \mu\text{m}$  (for this particular example). Thus, the core volumetric flow rate for these conditions is calculated to be  $5 \times 10^{-4}$  ml/s. This is a key quantity because it determines the total analysis time. For example, if the sample has a volume of one ml, the total analysis time is predicted to be 2,000 seconds.

However, I anticipate that my novel TDI/CCD flow cytometer will provide several improvements that will significantly decrease the analysis time:

1           1.    Image demagnification

2           Since the TDI approach provides an improvement in the  
3   signal-to-noise ratio of about 100 times over traditional flow  
4   cytometry, image demagnification can be used to lengthen the  
5   time that a target particle will be detected on a given pixel.  
6   Assuming a demagnification of 10, the maximum flow rate of  
7   25 cm/s from our discussion, above, becomes 250 cm/s. This  
8   then decreases the analysis time for a fixed flow volume by a  
9   factor of 10. While this approach also degrades the signal-  
10   to-noise ratio it is practical due to the improved signal-to-  
11   noise ratio achieved through my novel TDI approach.

12           2.    Increased core diameter

13           If the image is demagnified by a factor of 10, then the  
14   core diameter can be increased to about 90  $\mu\text{m}$  (for a CCD chip  
15   with 9  $\mu\text{m}$  pixels). Compared with a 50  $\mu\text{m}$  core diameter, this  
16   increases the sample volumetric flow rate by a factor of 3.2.  
17   Accordingly, the foregoing analysis time is decreased from  
18   2,000s to 625s.

19           3.    Faster CCD readout

20           A CCD camera that has read rates that are a factor of 10,  
21   or more, faster than the nominal read rate of 160 kHz can  
22   decrease the analysis time by an additional factor of 10, or  
23   more.

24           4.    Decreased spectral resolution

25           A two-color scheme for simultaneous detection of two  
26   species is possible. For example, using a spectral resolution



1 of 50 pixels (i.e., 512 lateral pixels binned to 50), would  
2 provide adequate spectral dispersion to resolve the two  
3 emission bands. The CCD can be binned to match the number of  
4 different types of target particles, e.g., using only four  
5 spectral elements, one for each emission band and two for the  
6 background signal. This change in spectral resolution by a  
7 factor of 10 changes the read time by a factor of about 10  
8 which decreases the analysis time accordingly.

9  
10 Clearly, of course, these four changes would not provide an  
11 aggregate decrease in the analysis time of 3,200 times. In  
12 particular, these changes either decrease the transit time of the  
13 target particle or increase the background noise which degrades the  
14 signal-to-noise ratio. However, one does have the latitude to  
15 increase the analysis speed by at least an order of magnitude and  
16 still have an order of magnitude increase in the signal-to-noise  
17 ratio over a conventional flow cytometer. Accordingly, I presented  
18 the above discussion to illustrate the point that the projected  
19 sampling time can be reduced by at least an order of magnitude.

20 Importantly, although I have focused this discussion of the  
21 use of TDI for the detection of target particles such as  
22 microorganisms using fluorescent labels, this novel discovery is  
23 not limited to microorganisms or to fluorescent measurements. For  
24 example, this same technique can be applied to the detection of  
25 single molecules through fluorescence or absorption spectroscopy in  
26 a flow cytometer. This is possible because TDI significantly

1 enhances the signal-to-noise ratio of the detector by decreasing  
2 the contribution of the background component from the surrounding  
3 medium. Examples of such molecular analysis are the presence of  
4 explosive molecules or groundwater contaminant molecules, e.g.  
5 benzene, trichloroethylene, etc.

#### 6 7 Detailed Description

8 Referring now to Figure 1, a first preferred embodiment of the  
9 novel TDI/CCD flow cytometer of this invention is shown generally  
10 at 10 and includes a laser 12, a flow zone 14 having a sample flow  
11 28 passing therethrough, reimaging optics 16 having a notch filter  
12 18 therein, an aperture 20, a focus lens 22 and a CCD 24. Laser 12  
13 emits a laser beam 26 through flow zone 14 to illuminate a first  
14 target particle 32 and a second target particle 33 (Figure 2)  
15 therein and to cause the same to fluoresce as fluorescence 30.  
16 Fluorescence 30 is optically processed by reimaging optics 16 and  
17 notch filter 18 and focused onto a field-of view aperture 20.  
18 Focus lens 22 focuses the resultant fluorescence 30 onto CCD 24  
19 (Figure 3) as will be discussed more fully hereinafter.

20 Sample flow 28 passes through flow zone 14 and exhibits a  
21 typical, parabolic-like flow velocity profile 29 as shown  
22 schematically herein by the dashed line in flow zone 14. Flow  
23 velocity profile 29 is created as a result of the surface drag  
24 phenomena imparted to the liquid of sample flow 28 by the wall  
25 surface of flow zone 14. This is an inherent feature of any liquid  
26 stream flowing through a conduit and can be readily eliminated by

1 the conventional technique of enclosing sample flow 28 in a sheath  
2 of moving air or liquid (not shown) to thereby create what is  
3 commonly referred to as a "sheath flow."

4 Referring now also to Figure 2, flow zone 14 with an  
5 irradiation zone 40 shown therein is shown greatly enlarged for  
6 ease of illustration. For example, laser beam 26 is shown as  
7 a 200 x 50  $\mu\text{m}$  laser beam illuminating flow zone 14 which is shown  
8 as 50 $\mu\text{m}$  diameter sample stream having a cylindrical cross section.  
9 A first target particle 32 is shown in sample flow 28 toward the  
10 lower end of irradiation zone 40 while a second target particle 33  
11 is shown entering the upper end of irradiation zone 40. A  
12 calibration particle 48 is also included in sample flow 28 for the  
13 purpose of allowing the operator (not shown) to suitably calibrate  
14 TDI/CCD flow cytometer 10 as will be discussed more fully  
15 hereinafter. Calibration particle 48 emits a calibration photon 49  
16 when illuminated by laser beam 26.

17 Referring now also to Figure 3, a greatly enlarged, schematic  
18 of a portion of CCD 24 is shown. Two shaded areas are shown as  
19 first pixel 42 and second pixel 43 which represent fluorescence 30  
20 from first target particle 32 and second target particle 33,  
21 respectively. First movement arrow 44 on first pixel 42 represents  
22 the movement or transfer of first pixel 42 across the face of CCD  
23 24. Correspondingly, second movement arrow 45 on second pixel 43  
24 represents the movement or transfer of second pixel 43 across the  
25 face of CCD 24. Importantly, the rate of movement of first  
26 movement arrow 44 and second movement arrow must be nearly

1 identical to that of sample flow 28 as imaged onto CCD 24 in order  
2 to minimize streaking of the digitized image obtained by CCD 24.  
3 The image of calibration particle 48 is not illustrated on CCD 24  
4 for purposes of simplicity although it would be essentially  
5 identical to that of either first pixel 42 or second pixel 43.

6 Referring to all of Figures 1-3, sample flow 28 passes through  
7 flow zone 14 carrying target first particle 32 and second target  
8 particle 33 therein along with an occasional calibration particle  
9 48. Laser 12 illuminates first target particle 32, second target  
10 particle 33, and calibration particle 48 with laser beam 26 as they  
11 each pass through irradiation zone 40 thereby causing first target  
12 particle 32, second target particle 33, and calibration particle 48  
13 to fluoresce due to intrinsic fluorescence (e.g. autofluorescence  
14 or bioluminescence) or due to being tagged with chemical labels  
15 that have characteristic emission wavelengths. Alternatively,  
16 first target particle 32, second target particle 33, and  
17 calibration particle 48 can cause characteristic scattering of  
18 laser beam 26. In any event, each of first target particle 32,  
19 second target particle 33, and calibration particle 48 emit  
20 specific illumination which is referred to herein as fluorescence  
21 30 for sake of simplicity or fluorescence 49 in the case of  
22 calibration particle 48. Fluorescence 30 along with fluorescence  
23 49 is optically processed in reimaging optics 16 containing a notch  
24 filter 18 therein. Notch filter 18 provides a filtering function  
25 by filtering out a significant portion of any background scattering  
26 caused by laser beam 26 generating scattering 50 from the carrier

1 fluid of sample flow 28 and even from the sidewalls of flow zone  
2 14, itself. Scattering 50 is shown as having a plurality of  
3 scattering rays 50a-50c which, in the absence of notch filter 18,  
4 would be picked up by reimaging optics 16 and directed to CCD 24.  
5 As it is presently constructed, notch filter 18 strongly attenuates  
6 light at the wavelength of laser beam 26 while permitting light of  
7 the wavelengths of fluorescence 30 and fluorescence 49 to pass.  
8 This is an important feature in flow cytometry since scattering  
9 rays 50 are noise which could significantly overwhelm the signal  
10 from first target particle 32 and second target particle 33 as well  
11 as from calibration particle 48.

12 Fluorescence 30 is directed through aperture 20 by reimaging  
13 optics 16 and focused by focus lens 22 onto CCD 24. In CCD 24,  
14 fluorescence 30 from first target particle 32 is recorded as first  
15 pixel 42 while fluorescence 30 from second target particle 33 is  
16 recorded as second pixel 43. First target particle 32 traverses  
17 flow zone 14 in sample flow 28 while first pixel 42 traverses the  
18 face of CCD 24 at (ideally) an identical rate of travel (assuming  
19 an image magnification of unity) as shown by first movement arrow  
20 44. Correspondingly, second pixel 43 representing second target  
21 particle 33 moves across the face of CCD 24 at the same rate of  
22 travel as second target particle 33 in sample flow 28 and as shown  
23 by second movement arrow 45.

24 As set forth previously, sample flow 28 (in a flow zone 14  
25 versus a sheath flow (not shown)) has a flow velocity profile 29.  
26 As such, the distance of each of first target particle 32, second

1 target particle 33, and calibration particle 48 from the axis of  
2 sample flow 28 will be represented by a differential in the  
3 velocity of flow of the respective particle. Accordingly, the  
4 rates of movement of first movement arrow 44 and second movement  
5 arrow 45 will be different as a result of the spatial separation of  
6 the respective target particle, first target particle 32 and second  
7 target particle 33, from the side wall of flow zone 14.  
8 Accordingly, this differential in the rates of movement of first  
9 target particle 32 and second target particle 33 will result in  
10 streaking of one or both of their images as recorded on CCD 24 as  
11 first pixel 42 and second pixel 43, respectively. However, by the  
12 application of relatively straight-forward statistical analysis  
13 techniques one can readily compensate for this flow phenomena  
14 caused by flow velocity profile 29. Further, one can use this same  
15 statistical analysis tool to provide a method for determining the  
16 rate of flow for a majority of first target particle 32 and second  
17 target particle 33 in flow stream 28 to thereby effectively control  
18 the rate of transfer of first pixel 42 and second pixel 43,  
19 respectively, across the face of CCD 24. This technique would be  
20 an effective way to eliminate the requirement for the use of  
21 calibration particle 48. Streaking of first pixel 42 or second  
22 pixel 43 is a relatively minor problem since a substantial portion  
23 of the flow of sample flow 28 is at or near the axis of flow as can  
24 be seen from flow velocity profile 29. Thus, while flow at the  
25 wall will have zero velocity, one can set the charge transfer rate  
26 on CCD 24 by measuring the streak length of target particles near

1 the axis of the flow. The enhancement of the signal-to-noise of  
2 fluorescence 30 through TDI results in a corresponding diminution  
3 of the effect of the signal from scattering ray 50b, particularly  
4 since scattering rays 50 tend to be distributed throughout sample  
5 flow 28 whereas fluorescence 30 is localized which results in a  
6 very low amount of signal from scattering ray 50b being recorded by  
7 CCD 24 in the same pixel, first pixel 42 or second pixel 43, as  
8 representative of the respective target particle, first target  
9 particle 32 or second target particle 33.

10 Referring now to Figure 4, a second embodiment of my novel  
11 TDI/CCD flow cytometer is shown generally at 60 and includes a  
12 laser 62, a flow zone 64, reimaging optics 66 with a notch filter  
13 68 therein, a slit 70, a collimator 72, a grating 74, a focusing  
14 lens 76, and a CCD 78. Laser 62 directs a laser beam 80 through  
15 flow zone 64 causing a first target particle 82 and a second target  
16 particle 83 (Figure 5) to fluoresce or otherwise emit fluorescence  
17 photons 84. Fluorescence photons 84 are optically processed  
18 through imaging optics 66 and notch filter 68 and are focused  
19 through aperture slit 70 and then collimated by collimator 72 and  
20 directed against grating 74. Grating 74 is designed to separate  
21 different wavelengths of fluorescence photons 84 prior to directing  
22 the same to CCD 78 as will be discussed more fully hereinafter.

23 Sample flow 88 also includes a flow velocity profile 89  
24 similar to flow velocity profile 29 (Figure 1) so that the  
25 discussion thereof would be identical to that previously discussed,

1 particularly with respect to using statistical analysis of sample  
2 flow 88 as a means for calibrating the readout of CCD 78.

3 Referring now to Figures 5 and 6, a portion of flow zone 64 is  
4 shown greatly enlarged and as having two different types of target  
5 particles therein, first target particle 82 and second target  
6 particle 83. Flow zone 64 is illuminated by laser beam 80 which  
7 causes first target particle 82 to emit a first fluorescence 96 and  
8 second target particle 83 to emit a second fluorescence 97 as well  
9 as a scattering fluorescence 90a-90c. In this particular  
10 embodiment of TDI/CCD flow cytometer 60, a first band 94 across  
11 flow zone 64 and containing first target particle 82 is read by CCD  
12 78 as a first spectrum 102 while a second band 95 across flow zone  
13 64 and containing second target particle 83 is read by CCD 78 as a  
14 second spectrum 103.

15 Sample flow 88 also includes a calibration particle 98 which  
16 emits a calibration photon 99 when illuminated by laser beam 80.  
17 Calibration particle 98 allows the operator (not shown) of TDI/CCD  
18 flow cytometer 60 to calibrate the operation of CCD 78 to the flow  
19 rate of sample flow 88 as will be discussed therein before with  
20 respect to target particle 48 (Figure 2).

21 First spectrum 102 moves across the face of CCD 78 at a rate  
22 illustrated schematically by first movement arrow 104 while second  
23 spectrum 103 moves across the face of CCD 78 at a rate illustrated  
24 schematically by second movement arrow 105. The rate of travel of  
25 first movement arrow 104 and second movement arrow 105 are each  
26 carefully coordinated with the rate of travel of sample flow 88 as



1 determined through the use of calibration particle 98. This is  
2 readily accomplished by detecting calibration photon 99 on CCD 78  
3 and coordinating its rate of movement across CCD with that of  
4 sample flow 88.

5 First spectrum 102 is shown having a particular cross hatch  
6 while second spectrum 103 is shown with a different cross hatch to  
7 illustrate that the spectra, first spectrum 102 and second spectrum  
8 103, have emissions at different wavelengths since first target  
9 particle 82 is a different species from that of second target  
10 particle 83. Further, the location of first target particle 82 can  
11 be at any position inside first target zone 92 while the location  
12 of second target particle 83 can be at any position inside second  
13 target zone 93 since both first target zone 92 and second target  
14 zone 93 are read out as first fluorescence 96 and second  
15 fluorescence 97, respectively. First fluorescence 96 is recorded  
16 by CCD 78 as first spectrum 102 while second fluorescence 97 is  
17 recorded by CCD as second spectrum 103.

18 The present invention may be embodied in other specific forms  
19 without departing from its spirit or essential characteristics.  
20 The described embodiments are to be considered in all respects only  
21 as illustrative and not restrictive. The scope of the invention  
22 is, therefore, indicated by the appended claims rather than by the  
23 foregoing description. All changes which come within the meaning  
24 and range of equivalency of the claims are to be embraced within  
25 their scope.

1       What is claimed and desired to be secured by United States  
2 Letters Patent is:

1  
2       1.   An apparatus for identifying a target particle  
3 comprising:

4           a sample stream containing said target particle;

5           a flow zone for said sample stream;

6           illumination means for illuminating said flow zone  
7 thereby illuminating said target particle in said sample  
8 stream as said target particle passes through said flow zone;

9           detection means for detecting illumination from said  
10 target particle; and

11          time delay integration means for integrating said  
12 illumination over a preselected interval of time.

1       2.   The apparatus defined in claim 1 wherein said sample  
2 stream is contained in a flow cytometer.

1       3.   The apparatus defined in claim 1 wherein said  
2 illumination means comprises a laser means.

1       4.   The apparatus defined in claim 3 wherein said laser means  
2 comprises a laser selected from the group consisting of a gas laser  
3 and a solid state laser.

1           5.    The apparatus defined in claim 1 wherein said sample  
2 stream includes a tag for tagging said target particle, said tag  
3 causing said target particle to emit said illumination for  
4 identifying said target particle.

1           6.    The apparatus defined in claim 1 wherein said sample  
2 stream includes a calibration particle for calibrating the rate of  
3 flow of said sample stream with said time delay integration means.

1           7.    The apparatus defined in claim 1 wherein said time delay  
2 integration means includes a CCD means for increasing registration  
3 of said illumination from said target particle while decreasing  
4 registration of scattered light.

1           8.    The apparatus defined in claim 7 wherein said time delay  
2 integration means includes statistical analysis means for analyzing  
3 a flow profile of said sample stream for controlling said time  
4 delay integration means with the rate of flow of said sample  
5 stream.

1           9.    The apparatus defined in claim 1 wherein said sample  
2 stream includes at least a first target particle and a second  
3 target particle, said first target particle emitting a first  
4 illumination having a first wave length and said second target  
5 particle emitting a second illumination having a second wavelength.

1        10. An apparatus for enhanced detection of a target particle  
2 in a sample stream comprising:  
3            a flow zone for said sample stream;  
4            radiation means for radiating said target particle in  
5 said sample stream as said sample stream passes through said  
6 flow zone, said radiation means causing an illumination from  
7 said target particle;  
8            optical means for optically processing illumination from  
9 said target particle;  
10            CCD means for registering said illumination optically  
11 processed by said optical means; and  
12            integration means for integrating said illumination  
13 registered by said CCD means.

1        11. The apparatus defined in claim 10 wherein said flow zone  
2 is included in a flow cytometer.

1        12. The apparatus defined in claim 10 wherein said sample  
2 stream includes a calibration particle for calibrating said TDI of  
3 said CCD.

1        13. The apparatus defined in claim 10 wherein said  
2 integration means includes statistical analysis means for analyzing  
3 the velocity of said target particles in said sample stream for  
4 calibrating said TDI of said CCD relative to the flow rate of said  
5 sample stream.

1        14. The apparatus defined in claim 10 wherein said sample  
2 stream includes at least a first target particle having a first  
3 spectrum and a second target particle having a second spectrum,  
4 said CCD means registering said first spectrum and said second  
5 spectrum.

1        15. A method for detecting a target particle in a sample  
2 stream comprising the steps of:

3            preparing a flow zone for said sample stream;  
4            flowing said sample stream with said target particle  
5 through said flow zone;

6            illuminating said target particle with a first  
7 illumination from an illumination source, said target particle  
8 emitting a second illumination;

9            detecting said second illumination from said target  
10 particle with a detector to produce a signal; and

11           integrating said signal over a period of time thereby  
12 increasing the signal-to-noise ratio of said signal from said  
13 second illumination from said target particle.

1        16. The method defined in claim 15 wherein said detecting  
2 step comprises recording said illumination with a CCD.

1           17. The method defined in claim 16 wherein said recording  
2 step includes analyzing said sample stream using statistical  
3 analysis means for coordinating said integrating step with the rate  
4 of flow of said sample stream.

1           18. The method defined in claim 15 wherein said flowing step  
2 includes incorporating a calibration particle in said sample stream  
3 thereby providing calibration for said integrating step.

1           19. The method defined in claim 15 wherein said flowing step  
2 include placing more than one tag in said sample stream, said tags  
3 thereby providing more than one of said second illumination.

1           20. The method defined in claim 15 wherein said preparing  
2 step includes forming said flow zone in a flow cytometer.

1 / 3

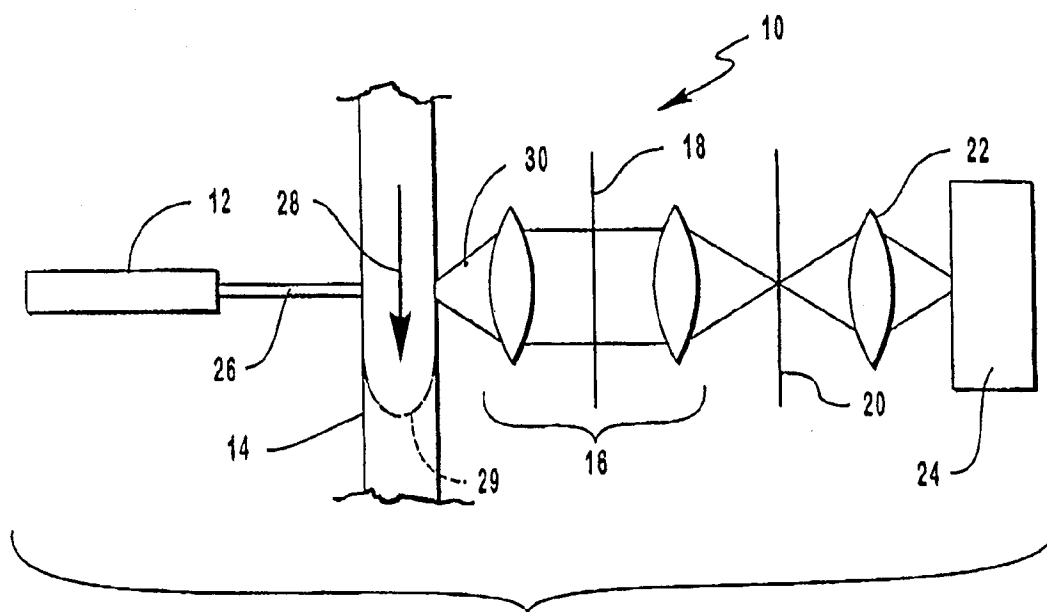


FIG. 1

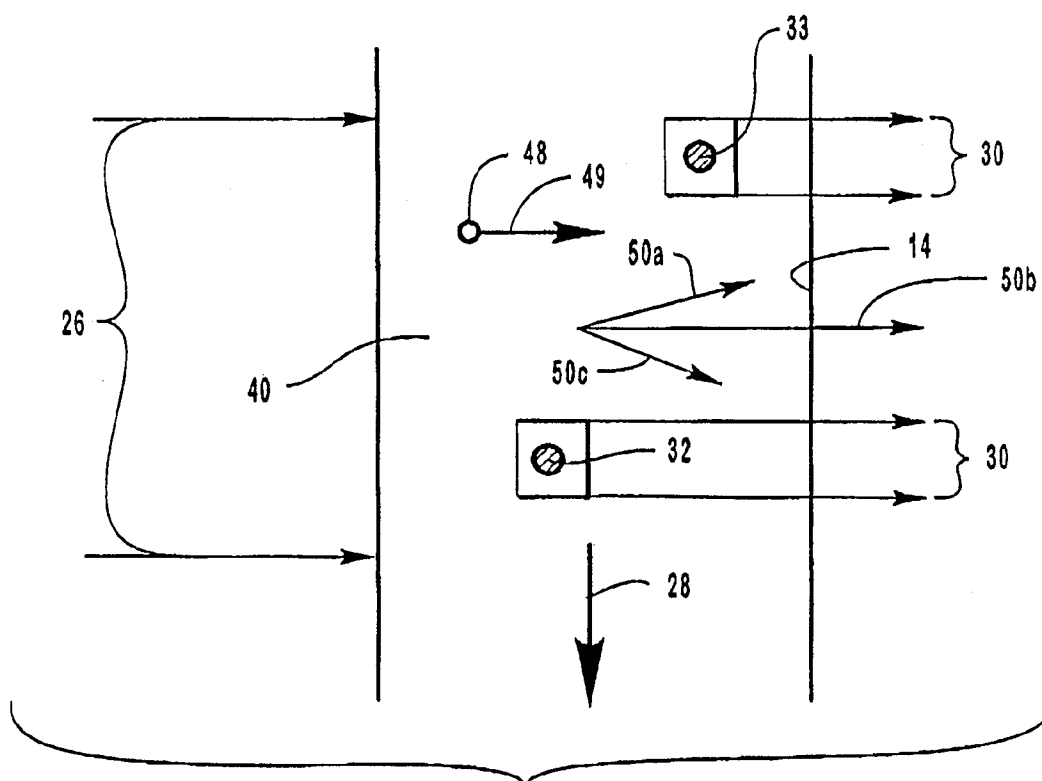


FIG. 2

2 / 3

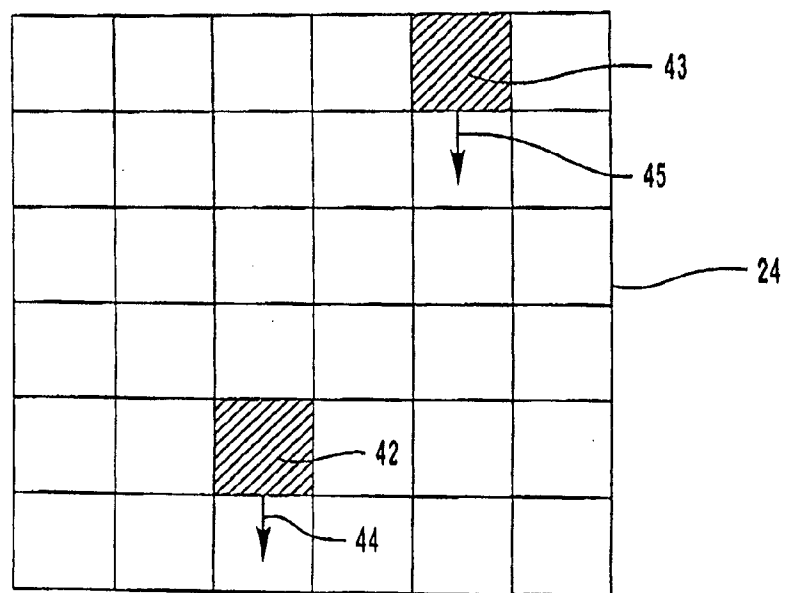


FIG. 3

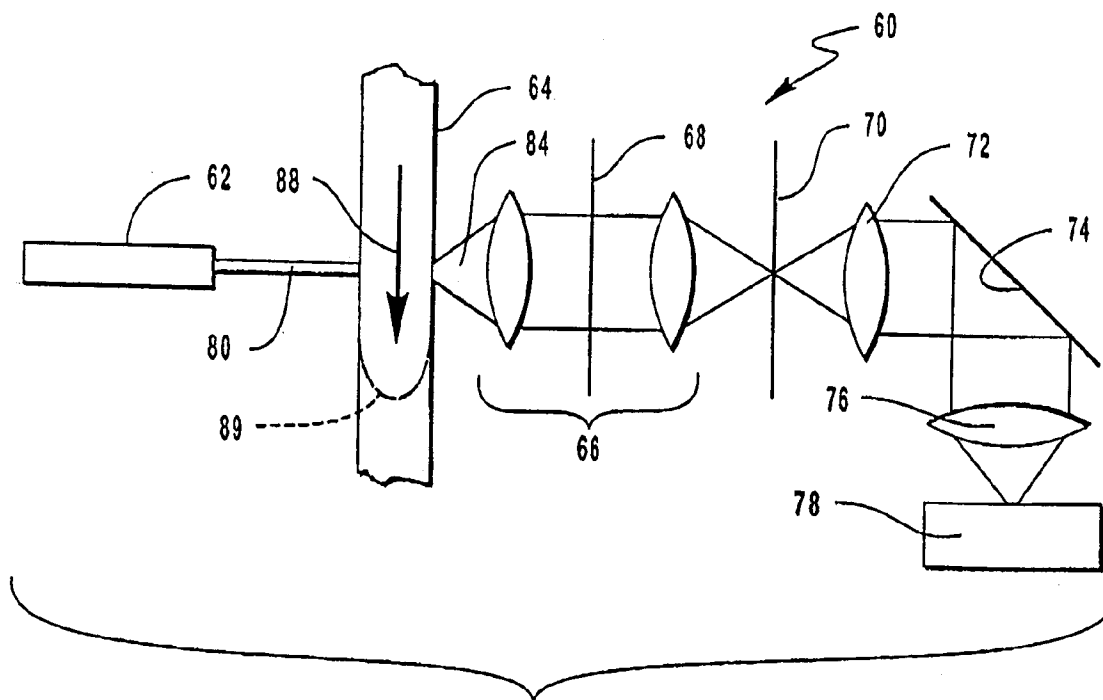


FIG. 4



3 / 3

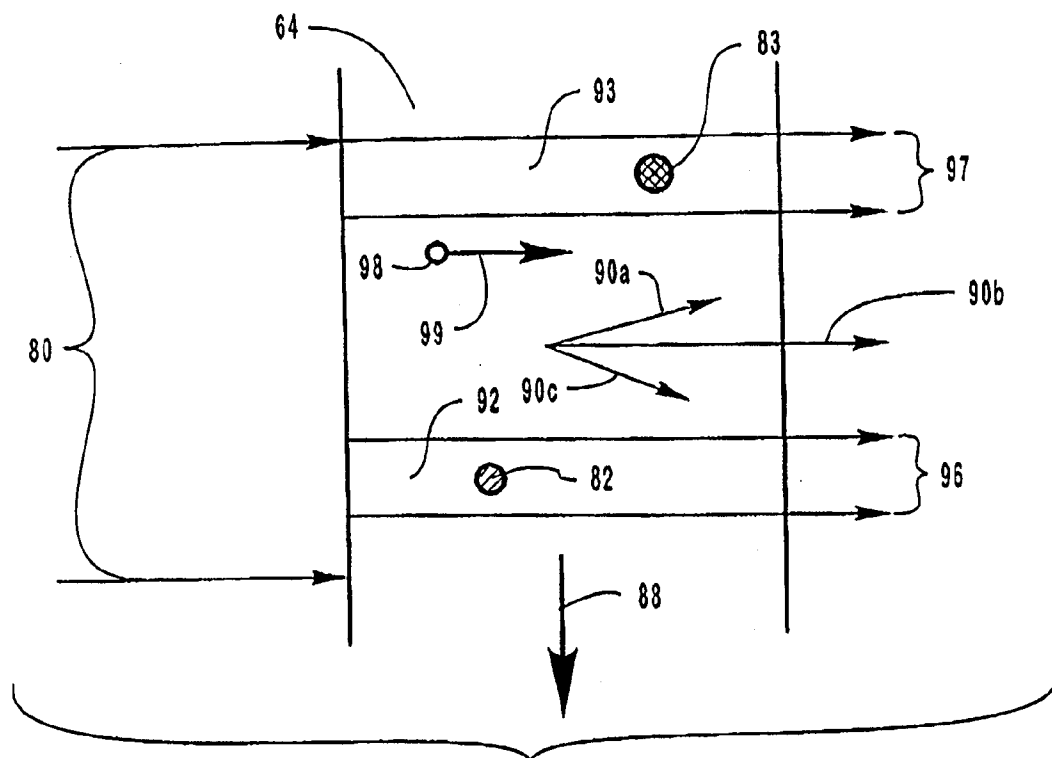


FIG. 5

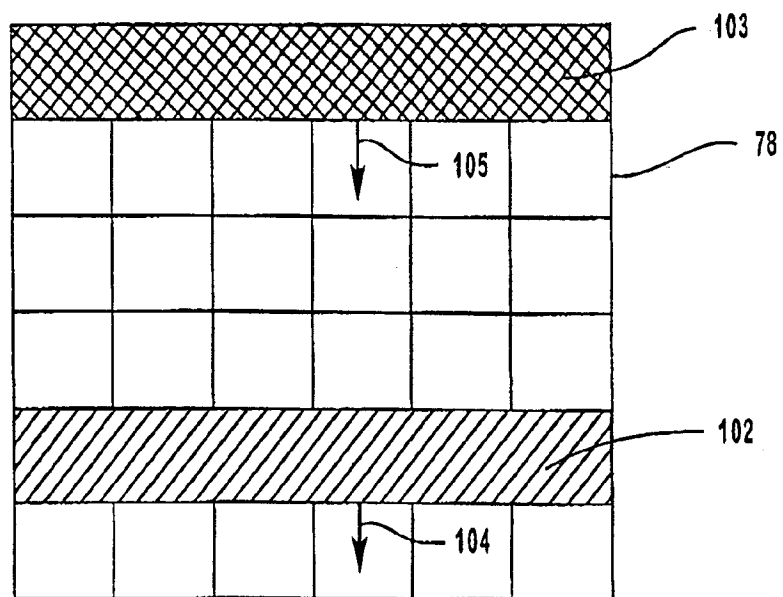


FIG. 6

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/00573

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 15/02

US CL :356/335

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 356/335-343, 73, 317, 39, 72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

US PTO APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,444,527 A (KOSAKA) 22 August 1995 (22-08-1995), entire document.	1-20
Y	US 5,674,743 A (ULMER) 07 October 1997 (07-10-1997), entire document.	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 MARCH 2000

Date of mailing of the international search report

17 APR 2000

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Frank G. Font

Telephone No. (703) 308-4881